ATTACHMENT TO AMENDMENT AND REPLY PURSUANT TO 37 C.F.R. §1.111
DATED JUNE 17, 2002

EXHIBIT 3

HUMAN GENE THERAPY 13:177–185 (January 20, 2002)
Mary Ann Liebert, Inc.

Systemic Administration of Naked DNA Encoding Interleukin 12 for the Treatment of Human Papillomavirus DNA-Positive Tumor

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ABSTRACT

Interleukin 12 (IL-12) is one of the most effective and promising cytokines for cancer therapy. Its therapeutic effects have been demonstrated in a variety of tumors in animal models when it is administrated locally or systemically. We describe here a systemic delivery of naked murine IL-12 (mIL-12) gene *in vivo*. Dose-dependent systemic production of mIL-12, with a serum level up to approximately 20 μg/ml, was observed 24 hr after systemic gene delivery. The apparent half-life in the circulation was about 5 hr. The result of a bioactivity assay (*in vitro* interferon γ [IFN-γ] release) indicated that the gene product in mice was as active as the purified recombinant murine IL-12 protein (rmIL-12). The circulating mIL-12 activated natural killer cells and stimulated IFN-γ production *in vivo*. A single administration of mIL-12 gene resulted in prominent regression of established subcutaneous tumor in a human papillomavirus (HPV) DNA-positive tumor model (TC-1) in C57BL/6J mice. The antitumor effect of the single gene dose was comparable to repeated intraperitoneal administration of rmIL-12 (0.5 μg/day for consecutive 5 days). This systemic gene delivery is simple, economical, and highly efficient for the production of large amounts of cytokine *in vivo*. With this gene delivery method, we have demonstrated the therapeutic potential of IL-12 for the treatment of HPV DNA-positive tumor and the usefulness of the systemic gene delivery for assessing the therapeutic effect of a candidate gene.

OVERVIEW SUMMARY

By employing hydrodynamics-based liver gene delivery of the naked mIL-12 gene, we have observed the production of high-level secretable murine IL-12 (mIL-12) in mice. The level of mIL-12 in serum was dependent on gene dose and declined with time. The gene product was functionally active *in vitro*, with bioactivity comparable to that of purified recombinant mIL-12 protein for the treatment of HPV DNA-positive tumor in an animal model.

INTRODUCTION

Functionally active interleukin 12 (IL-12) is a heterodimer (p70) composing of two subunits (p35 and p40) encoded by two different genes. The two subunits are covalently linked by a disulfide bond to form an active cytokine molecule. The homodimer of p40 is antagonistic to the activity of IL-12 p70 (Gillessen *et al.*, 1995). IL-12 has been shown to have many different biological activities, including the induction of interferon γ (IFN-γ) production from natural killer (NK) cells and T cells, induction of type 1 T helper (Th1) cell maturation (Manetti *et al.*, 1993), the ability to enhance NK and cytotoxic T lymphocyte (CTL) activities, regulation of the production of many other cytokines, and stimulation of hematopoietic stem cell proliferation (Jacobsen *et al.*, 1993; Brunda, 1994; Wolf *et al.*, 1994). Owing to its many beneficial biological activities, IL-12 has been evaluated as a therapeutic agent for the treatment of cancer, infectious disease, and allergic disorders.

IL-12 has been demonstrated as one of the most potent antitumor cytokines in a number of tumor models, including cancers of the breast, kidney, ovary, lung, and brain, as well as fibrosarcoma and melanoma (Dias *et al.*, 1998; Sumimoto *et al.*, 1998). For most studies, local or systemic administration (by the intraperitoneal route) of recombinant IL-12 protein or IL-12 gene was used. The systemic immunostimulating effects of this cytokine make it attractive for the treatment of metastases or distant tu-

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mors (Mu *et al.*, 1995). Moreover, IL-12 confers protective activity in vaccination studies (Rodolfo *et al.*, 1996; Sun *et al.*, 1998).

Human papillomavirus (HPV) infection is known to induce tumorigenesis. Infection with HPV, especially type 16, has been shown to be associated with cervical cancer, the second most common cancer in women, and it causes about half a million deaths each year worldwide. HPV type 16 infection is a high-risk factor for cervical carcinoma (Josefsson *et al.*, 2000), squamous cell carcinoma of the head and neck (Sudbo, 2001), bladder transitional cell carcinoma (Chen *et al.*, 2000), and esophageal cancer (Li *et al.*, 2001). Therefore, development of therapy for treatment of HPV DNA-positive tumor becomes essential. Vaccination and antisense approaches targeting HPV oncoproteins have shown some success in some experimental models. Vaccination with IL-12 and HPV E6 DNA resulted in an enhanced antitumor response (Tan *et al.*, 1999). Our earlier observation that local administration of liposome–IL-12 DNA complexes could result in a partial antitumor response (which was synergized by E7 antisense in cervical cancer treatment) demonstrated that IL-12 cytokine therapy might be effective for the treatment of various cancers carrying the HPV genome (He *et al.*, 1998).

Owing to its promising therapeutic effects, many different approaches were employed for the delivery of IL-12, including purified recombinant protein and *ex vivo* delivery of engineered fibroblasts (Kang *et al.*, 1999). Viral vectors for IL-12 gene delivery have been explored, which include adenovirus construct (Siders *et al.*, 1998), retroviral vector (Tahara *et al.*, 1994), herpes simplex virus (Parker *et al.*, 2000), canarypox virus vector (Puisieux *et al.*, 1998), and adeno-associated virus (Paul *et al.*, 2000). For nonviral methods, particle bombardment of naked IL-12 DNA coated on gold beads (i.e., the gene gun) has resulted in the eradication of various types of established primary and metastatic tumors (Rakhmilevich *et al.*, 1997). Moreover, intradermal injection of plasmid DNA (Tan *et al.*, 1996) and intratumor injection of liposome–murine IL-12 (mIL-12) DNA complex have also resulted in some antitumor effects (He *et al.*, 1998). For the administration of a cytokine gene, nonviral gene delivery may be more advantageous as it induces a minimal immune response compared with viral vectors. However, at this moment, the efficiency of gene delivery by most nonviral methods is still lower than that of viral methods.

In this study, we took advantage of an efficient systemic nonviral gene delivery method, hydrodynamics-based liver gene delivery (Liu *et al.*, 1999), for *in vivo* mIL-12 gene transfer. Functional comparison of the gene product and the purified recombinant mouse IL-12 protein (rmIL-12) was carried out. We also investigated the antitumor effect of systemic IL-12 gene transfer in a subcutaneous HPV DNA-positive tumor model in comparison with rmIL-12. Our results demonstrated that mIL-12 gene therapy was effective for the treatment of an HPV DNA-positive tumor in an animal model.

MATERIALS AND METHODS

Plasmid constructs

Plasmids pNGVL3-mIL12 and pNGVL-1 were kind gifts from the National Gene Vector Laboratory (University of Michigan, Ann Arbor, MI). The pNGVL3-mIL12 plasmid carries the murine p35 gene, an internal ribosome entry site, and the p40 gene driven by a single cytomegalovirus (CMV) promoter, because coexpression of p35 and p40 genes in target cells is required to generate a biologically active p70 heterodimer (Gubler *et al.*, 1991; Schoenhaut *et al.*, 1992). The pNGVL-1 plasmid is a control plasmid, which contains the backbone sequence of pNGVL3-mIL12 without the mIL-12 insert.

Purified recombinant mIL-12 protein administration

Purified recombinant mIL-12 protein was a generous gift from the Genetics Institute (Cambridge, MA). rmIL-12 was administered (once per day for five consecutive days) into C57BL/6J mice by intraperitoneal injection in 150 μl of phosphate-buffered saline (PBS) containing 0.1% mouse albumin fraction V (Sigma, St. Louis, MO). Control mice were injected with PBS containing the same amount of mouse albumin fraction V.

Hydrodynamics-based liver gene delivery

Plasmid DNA was purified with a plasmid purification kit (Qiagen, Valencia, CA). Various amounts of DNA were diluted in 0.9% sterilized NaCl and injected intravenously into mice in a volume of 1.6 ml in about 8 sec as described (Liu *et al.*, 1999).

ELISA

Mice were bled from the tail vein at various time points after treatment. Serum samples were collected by coagulating the blood at room temperature (2 hr) or at 4°C (overnight), followed by high-speed centrifugation at 14,000 rpm, 4°C for 20 min. Quantification of mIL-12 was performed with an mIL-12 p70 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) for the detection of the heterodimer p70. The concentration of p70 in the serum samples was determined from a standard curve produced with known quantities of p70. Similarly, the production of IFN-γ *in vivo* was assayed with a mouse IFN-γ ELISA kit (R&D Systems). IFN-γ produced in the supernatant from splenocyte cultures was similarly quantified by ELISA.

NK activity (YAC-1 cell lysis)

Lymph nodes and spleens of C57BL/6J mice were harvested by aseptic technique. Cells from lymph nodes (inguinal, axillary, and mesenteric) and spleen were collected by meshing the tissues through a nylon mesh (70 μm). After centrifugation at 1500 rpm for 5 min, lymphocytes were resuspended in RPMI containing 1× minimal essential medium (MEM), 0.01 *M* HEPES, 10% heat-inactivated fetal bovine serum (FBS), and 1× antibiotics–antimycotics. Splenocytes were treated with 5 ml of red blood cell (RBC) lysis buffer (8.32 g of NH_4Cl, 0.84 g of $NaHCO_3$, and 0.043 g of EDTA in 1 liter of H_2O) for 5 min at room temperature. RBC lysis was neutralized by the addition of 10 ml of RPMI. Splenocytes were then centrifuged for 5 min at 1500 rpm and resuspended in RPMI. YAC-1 cells were radiolabeled with 150 μCi of ^{51}Cr (NEN DuPont, Boston, MA) for 2 hr at 37°C with swirling. Radiolabeled cells were then washed three times in 15 ml of RPMI. Labeled YAC-1 cells (2×10^4 cells/well) were incubated with lymphocytes at various ratios in a 96-well plate for 4–5 hr at 37°C. YAC-1 cells lysed by 0.1% sodium dodecyl sulfate (SDS) gave maximal release, whereas YAC-1 cells alone (without lymphocytes) gave minimal release. Triplicates or more were set up for each ratio.

After incubation, the plate was spun at 1500 rpm for 5 min and supernatant was collected for γ counting.

In vitro *IFN-γ release assay*

Spleens were collected from C57BL/6J mice by aseptic technique. Splenocytes were harvested in RPMI as described above and were seeded at a concentration of 5×10^6/ml in a 24-well plate. After the addition of mIL-12 at various concentrations, the splenocytes were cultured for 48 hr at 37°C in 5% CO_2. Supernatant was collected for IFN-γ quantification by ELISA.

Tumor model

TC-1 cells (cultured in RPMI), a mouse lung epithelial cell line transformed with the HPV-16 genome (Lin *et al.*, 1996), were inoculated subcutaneously at 1×10^5 cells/mouse in 6-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). TC-1 cells were trypsinized and washed three times in Hanks' buffer. At the final washing, cell number and viability were determined by trypan blue exclusion. Samples with greater than 80% viable cells were inoculated subcutaneously in the flank of each mouse. Tumors were allowed to establish for 9 days before the systemic delivery of naked DNA or rmIL-12 treatment. Tumor size was measured every 2–3 days and the volume of each tumor was calculated by the following equation: $^1/_2 \times$ width $\times$ length^2 (mm^3). Tumor growth was calculated as fractional tumor volume (volume of tumor compared with initial volume of each tumor).

RESULTS

Pharmacokinetics of rmIL-12 and mIL-12 gene expression

Pharmacokinetics of mIL-12 gene expression after systemic naked DNA delivery was compared with the pharmacokinetics of rmIL-12 administration after intraperitoneal injection of recombinant protein. Five micrograms of pNGVL3-mIL12 plasmid was injected into each mouse and serum samples were collected for the detection of circulating mIL-12 levels by ELISA. As reported previously (Liu *et al.*, 1999), gene expression mediated by hydrodynamics-based gene transfer was dependent on the speed of injection. Therefore, the speed of injection was carefully controlled (about 8 sec) for all experiments.

As shown in Fig. 1, at 60 min after injection, a low level of mIL-12 was detected in serum (about 0.2 ng/ml, $n = 5$). As short as 5 hr after injection, a rapid increase (about 18,000-fold) in the level of mIL-12 production (6.4 $\pm$ 1.3 μg/ml, $n = 5$) was observed. The expression of mIL-12 kept increasing. At 10 hr after injection, the level of mIL-12 production was 13.1 $\pm$ 3.2 μg/ml ($n = 4$) and then it reached a maximum level of 22.7 $\pm$ 2.7 μg/ml ($n = 4$) at about 17 hr. Systemic administration of the control pNGVL-1 plasmid caused a nonspecific production of mIL-12 at a low level (about 1 ng/ml at 5 hr) (data not shown). The result showed that liver gene transfer can efficiently produce high levels of mIL-12 *in vivo* and that the cytokine is secreted into the circulation. As shown in Fig. 2, the level of mIL-12 production did not decrease until 2 days after injection. There was an approximate 30-fold reduction in gene expression per day with any given dose of DNA. The apparent half-life of the circulating mIL-12 produced by the gene transfer was estimated to be about 5 hr.

For the pharmacokinetics study of rmIL-12 distribution, 600 ng of recombinant protein was injected intraperitoneally. Upon injection, a rapid distribution of rmIL-12 in the circulation was observed as soon as 10 min after injection, about 1.3% of the injected dose was detected in the circulation. At the peak of absorption (about 60 min), 20% of the injected dose was detected (88 ng/ml is equivalent to a total amount of 114 ng in mice with a blood volume of about 1.3 ml). Thereafter, the level of rmIL-12 started to decrease slowly with an apparent half-life (in the elimination phase) of about 205 min. The longer apparent half-life (300 min) of the mIL-12 produced by systemic gene de-

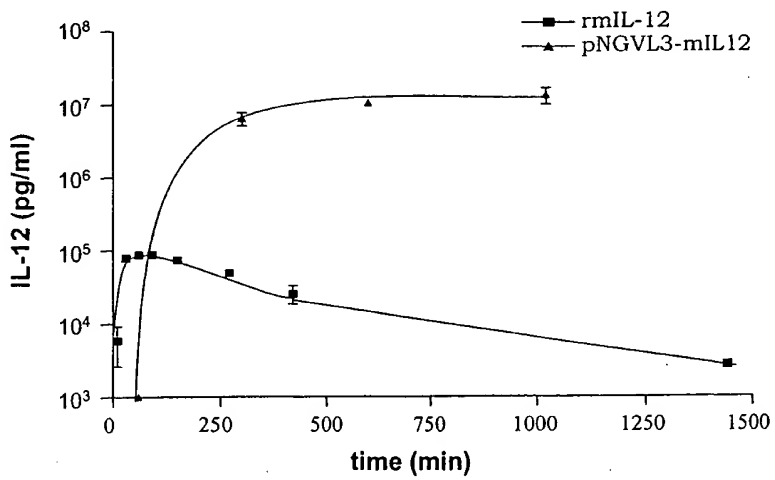

FIG. 1. Pharmacokinetics of recombinant mIL-12 protein (rmIL-12) and mIL-12 gene expression in C57BL/6J mice. Six hundred nanograms of rmIL-12 was injected intraperitoneally. Serum mIL-12 levels were determined 10, 30, 60, 90, 150, 270, and 420 min after injection ($n = 3$). Five micrograms of pNGVL3-mIL12 was administered to the mice by hydrodynamics-based liver gene delivery. Circulating mIL-12 level (p70 heterodimer) was measured by ELISA. Data are shown as means $\pm$ SEM ($n \geq 3$).

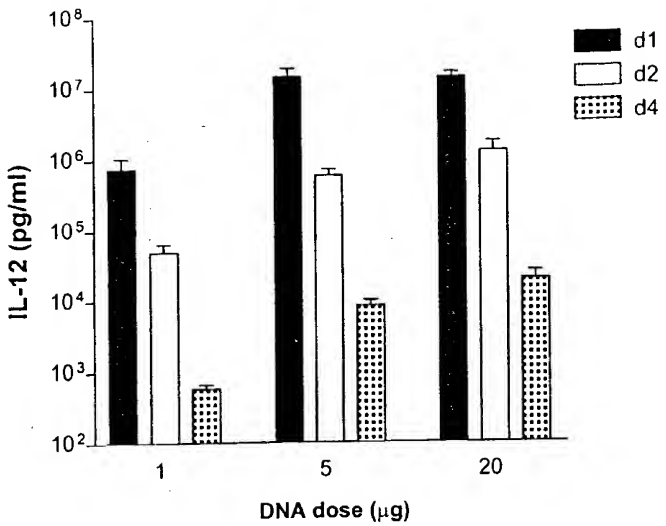

FIG. 2. A time course study of the effect of gene dose on the expression of mIL-12 by systemic delivery of naked DNA. Various amounts of pNGVL3-mIL12 (1, 5, and 20 μg) were injected into each group of mice ($n \geq 4$). The circulating level of mIL-12 was measured on days 1, 2 and 4 after gene delivery.

livery was probably due to the continuous production of mIL-12 by the liver after gene transfer.

Effect of gene dose on mIL-12 production

To investigate the effect of gene dose on the production of mIL-12, mice were injected intravenously with various doses of pNGVL3-mIL12 (1, 5, and 20 μg). In addition, the time course of expression was also monitored 1, 2, and 4 days after injection (Fig. 2). A general dose-dependent effect of mIL-12 was observed. However, the level of mIL-12 production seemed to reach a maximum with 5 μg of DNA on day 1, as there was no significant difference between the 5- and 20-μg DNA doses. A 13-fold difference in gene expression was observed between the 20- and 1-μg doses. As discussed previously, the level of circulating mIL-12 started to drop rapidly by day 2 (approximately 30-fold per day). By day 4, the level of mIL-12 was reduced to about 580, 8400, and 17,800 pg/ml for the groups treated with 1, 5, and 20 μg of DNA, respectively. With the highest gene dose, a level of about 1800 pg/ml was still detectable on day 7 (data not shown).

NK cell activation

IL-12 is known to enhance NK cell activity *in vivo* (Brunda, 1994). To study whether systemic gene delivery was able to produce functionally active mIL-12, an NK cell assay was performed. Cells from lymph nodes and spleens were collected from mice 6 days after gene transfer. Specific YAC-1 cell lysis was observed with lymph node cells collected from the pNGVL3-mIL12-treated group (Fig. 3). However, no detectable NK activity was observed with splenocytes collected from the same group of mice (data not shown). Repeated injection of rmIL-12 protein (0.5 μg/day for five consecutive days) resulted in weaker NK activation (5.7-fold lower than in the pNGVL3-mIL12 group) on day 6 after the last injection (data not shown). Lymph node cells from the pNGVL-1 control group showed only minimal background lysis of about 3%. This mIL-12-mediated NK cell activation demonstrated that the gene transfer could produce functionally active mIL-12 *in vivo*.

Stimulation of IFN-γ production in vivo

Besides the enhancement of NK activity, IL-12 is known to stimulate the production of IFN-γ both *in vivo* and *in vitro*. Moreover, IFN-γ is believed to be one of the major cytokines mediating the antitumor effects of IL-12. Therefore, the time course for the stimulation of IFN-γ production after IL-12 gene delivery was monitored and compared with that of rmIL-12 administration. Five micrograms of plasmid DNA, pNGVL3-mIL12 or pNGVL-1, or 1 μg of rmIL-12, was administered to mice. On day 1 after IL-12 gene transfer, only a minimally detectable level of IFN-γ (98.5 ± 15.7 pg/ml, $n = 3$) was produced even though the circulating mIL-12 level at this time was maximal (Figs. 4 and 2). Injection of pNVGL-1 control plasmid also produced a similarly low level of IFN-γ on day 1. For the pNGVL3-mIL12 group, the level of IFN-γ production rapidly increased by 84-fold (8.3 ± 1.1 ng/ml, $n = 3$) by day 2 and further increased up to 288-fold (28.4 ± 14.4 ng/ml, $n = 3$) by day 4. The circulating level of IFN-γ remained high up to day 7, even though the mIL-12 level was low at this time (Fig. 1). The control plasmid pNGVL-1 showed only minimal IFN-γ production (Fig. 4). In comparison with IL-12 gene transfer, administration of rmIL-12 protein resulted in only a rapid but transient stimulation of IFN-γ production. High levels of IFN-γ were produced on days 1 and 2 after injection (700.8 ± 100.5 and 537.3 ± 230.8 pg/ml, respectively). However, the IFN-γ level rapidly decreased to an undetectable level (0 ± 0 pg/ml) by day 4 (Fig. 4).

In vitro *IFN-γ production induced by mIL-12*

To compare the activity of mIL-12 produced in mice with the activity of purified rmIL-12, their abilities to induce IFN-γ

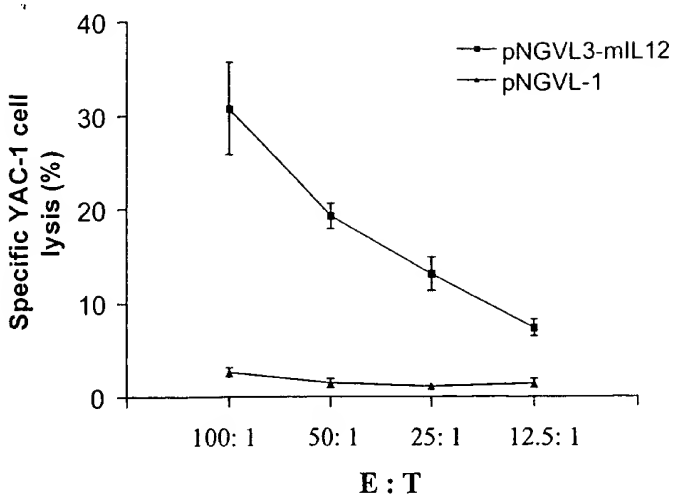

FIG. 3. Activation of natural killer (NK) cells by pNGVL3-mIL12 gene transfer. Specific lysis of YAC-1 cells from lymphocytes (collected from lymph nodes) of the treated mice was measured in a ^{51}Cr release assay on day 6 after gene delivery.

production in primary splenocyte cultures were measured. Circulating mIL-12 was obtained from the sera of mice 1 day after injection of pNGVL3-mIL12 and the concentration of mIL-12 in sera was estimated by ELISA. In the concentration range of 10-10,000 pg/ml, both the gene product in serum and rmIL-12 had similar, if not identical, sigmoidal dose–response curves in inducing the production of IFN-γ at 48 hr (Fig. 5). In an [^{3}H]thymidine incorporation assay (a common bioassay for IL-12), the mIL-12 gene product was also active in inducing the proliferation of splenocyte cultures (data not shown). The results further confirmed that systemic gene delivery could produce large amounts of functionally active mIL-12 that was indistinguishable from rmIL-12.

Antitumor effect of pNGVL3-mIL12

Because the gene delivery method produced large amounts of mIL-12 with a duration of expression of about 4–7 days, we investigated whether systemic mIL-12 gene delivery would show any therapeutic effect in an aggressive HPV DNA-positive tumor model. TC-1 cells were inoculated subcutaneously in C57BL/6J mice. Mice bearing established tumors were treated either with rmIL-12 (0.5 or 0.2 μg/day for five consecutive days) or with a single pNGVL3-mIL12 gene dose (5 μg) delivered by systemic administration. Control plasmid pNGVL-1 (5 μg) was administered to the control group. Growth of tumors (measured as fractional tumor volume = measured tumor

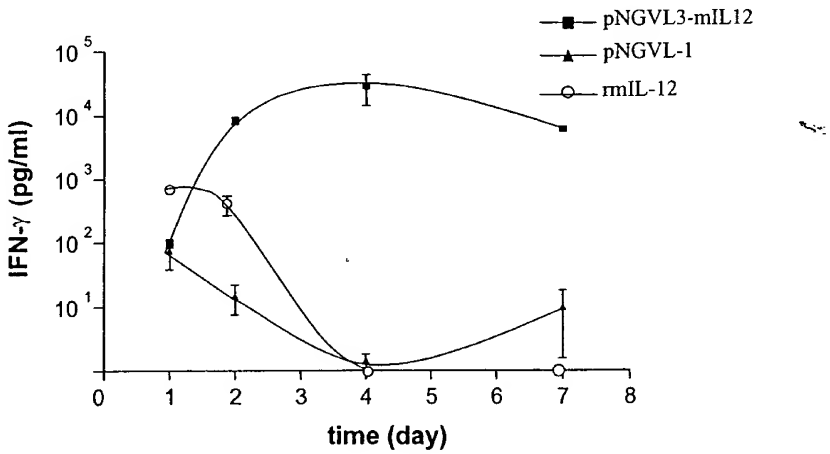

FIG. 4. Comparison of IFN-γ production *in vivo* after pNGVL3-mIL12 gene delivery or rmIL-12 administration. Mice (n = 3) were injected with a single dose of 5 μg of DNA or 1 μg of rmIL-12 and the level of IFN-γ in the serum on various days after injection was measured by ELISA. IFN-γ production stimulated by control pNGVL-1 DNA is also shown.

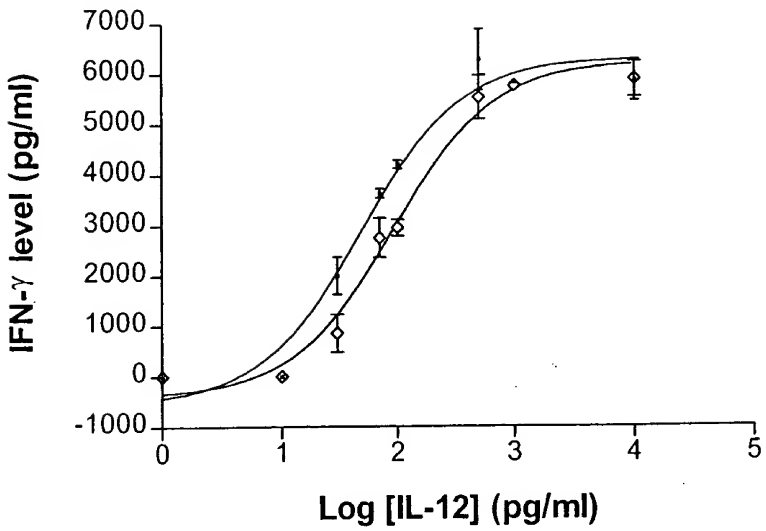

FIG. 5. Comparison of bioactivities of rmIL-12 and serum mIL-12 produced by pNGVL3-mIL12 injection. Splenocytes from untreated C57BL/6J mice were cultured for 2 days with various concentrations of rmIL-12 (solid squares) or serum mIL-12 (open diamonds) produced after *in vivo* gene transfer ($n = 3$). Supernatants of the splenocyte cultures were collected for the quantitation of IFN-γ production.

volume/initial volume of each tumor) in the different groups was monitored for 32 days (Fig. 6). Twenty-five days after tumor inoculation, mice treated with a single dose of pNGVL3-mIL12 had 100% tumor regression, which was comparable to the regression (89%) in the group of mice treated with rmIL-12 (0.5 μg/day for five consecutive days). However, for the mice treated with a lower dose of rmIL-12, a partial therapeutic effect was seen (63% regression on day 25, with later on some tumor reappearing, for a final regression of 25% on day 32). Mice treated with the control pNGVL-1 plasmid had no tumor regression at any time, with the mean (±SEM) tumor volume larger than that of all the mIL-12-treated groups. This

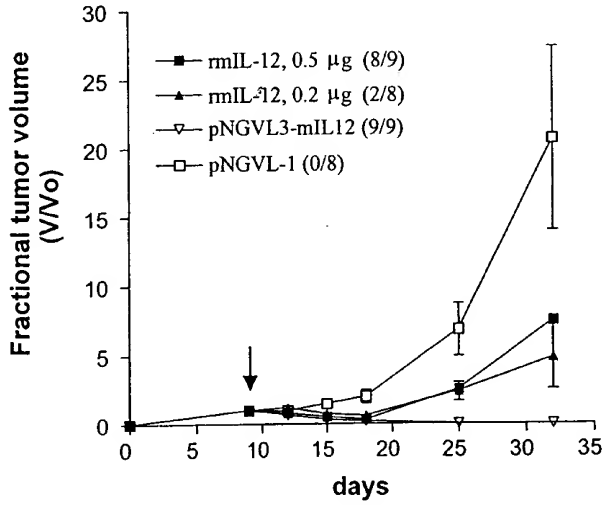

FIG. 6. Comparison of the antitumor effect of rmIL-12 administration and pNGVL3-mIL12 gene delivery. Subcutaneous TC-1 tumors were established in C57BL/6J mice. On day 9 after tumor inoculation, the mice in each group ($n \geq 8$) were treated with either rmIL-12 (0.5 or 0.2 μg, intraperitoneal, for five consecutive days) or with a single dose of 5 μg of pNGVL3-mIL12 or control pNGVL-1 plasmid by systemic naked DNA delivery (indicated by the arrow). The fractional tumor volume (measured tumor volume divided by the initial tumor volume) of the nonregressed tumors is shown. The numbers shown in parentheses indicate the number of tumors regressed/total number of tumors inoculated.

result showed that mIL-12 produced by hydrodynamics-based gene delivery was as potent as rmIL-12 for the treatment of the HPV DNA-positive tumor model in mice.

DISCUSSION

IL-12 is one of the best antitumor cytokines for cancer therapy, as it is known to have a variety of immunostimulating effects. Its antitumor activity has been demonstrated in a number of tumor models including breast, kidney, ovarian, lung, brain, melanoma, and fibrosarcoma (Dias *et al.*, 1998; Sumimoto *et al.*, 1998). However, the antitumor effect of IL-12 is not universal. Resistance to IL-12 therapy has been demonstrated in some acute myeloid leukemia and melanoma models (Nishimura *et al.*, 1996; Ladanyi *et al.*, 1998; Vitale *et al.*, 1998).

The antitumor effect of IL-12 for the treatment of HPV DNA-positive tumor has not been investigated in detail, but some studies implied the potential of such treatment. Skin transfection of IL-12 potentiates the human papillomavirus E6 DNA vaccine-induced antitumor immune response (Tan *et al.*, 1999). In addition, we demonstrated earlier that local administration (intratumor injection) of liposome–mIL-12 DNA complexes could cause partial regression of a less aggressive HPV DNA-positive tumor model (C3 cells) (He *et al.*, 1998), indicating the therapeutic potential of IL-12 for HPV DNA-positive cancers. In this study, we showed that high-level circulating IL-12 produced by a single gene transfer was sufficient for complete eradication of established subcutaneous tumors in an aggressive HPV DNA-positive tumor model (TC-1 cells). The antitumor effect was specific to mIL-12, as the administration of recombinant mIL-12 also resulted in a similar antitumor effect. However, repeated injection of a high dose (0.5 μg/day for five consecutive days) of rmIL-12 was needed to achieve an antitumor effect similar to that of single gene transfer. A lower dose of recombinant mIL-12 (0.2 μg/day for five consecutive days) resulted in only a partial antitumor response. This indicates that the dose of IL-12 is a determining factor for its antitumor potency. Although single injection of IL-12 resulted in complete tumor regression in this study, we have also evaluated the effectiveness of repeated injection (Lui *et al.*, 2001). We found that initial IL-12 expression resulted in the attenuation of expression of the subsequently delivered IL-12 gene or reporter gene. A refractory period was observed for the second gene dose to be effective. This was found to be mediated mainly by IFN-γ, which is a downstream effector of IL-12 (Lui *et al.*, 2001).

The production of mIL-12 by systemic gene transfer was accompanied by the induction of a high and sustained level of IFN-γ (compared with rmIL-12 administration) *in vivo* (Fig. 4). Although the mechanisms underlying the antitumor effects of IL-12 have not been fully elucidated, IFN-γ is believed to have an important role in mediating the effect (Brunda *et al.*, 1995). IFN-γ activates innate immunity such as the activation of NK cells and macrophages, which could help in eradicating tumor cells. Indeed, mIL-12 gene transfer resulted in the activation of NK cells collected from lymph nodes in our studies (Fig. 3). The reason for the undetectable activation of NK cells in lymphocytes collected from spleen is unknown. In addition, IFN-γ can act directly on T and B lymphocytes to promote differentiation. IFN-γ can also enhance both cellular and humoral immunity by inducing the expression of class I and II MHC molecules (Schendel *et al.*, 2000). In addition, the antitumor effect of IL-12 may also be enhanced by the antiangiogenic activity of IL-12 (Sunamura *et al.*, 2000). Additional mediators other than IFN-γ may be involved in the antitumor activity of IL-12, as the antitumor activity of IL-12 seems to be better and more consistent than that of IFN-γ (Paillard, 1998).

Our results showed that large amounts of functionally active mIL-12 could be produced by systemic naked DNA delivery. The gene product not only had a level of antitumor activity comparable to that of rmIL-12 (Fig. 6), but was also similar to rmIL-12 in terms of bioactivity (Fig. 5). In fact, the antitumor efficacy of this systemic naked DNA delivery is much higher than that of intratumor delivery of IL-12 DNA–liposome complex or the particle bombardment method, in which multiple gene transfer is needed to elicit complete tumor regression (our unpublished data). This is probably due to the high level of mIL-12 produced in the circulation and sustained gene expression, especially in the liver (Liu *et al.*, 1999).

In conclusion, our results suggested that the gene transfer method used in this study could be useful for rapid screening of IL-12-sensitive tumors, in addition to the HPV DNA-positive tumor model tested here. It would provide important information for the prediction of responsiveness of different types of cancer to IL-12 therapy.

We have also reported the *in vivo* production of Flt3 ligand by the same delivery approach (He *et al.*, 2000), resulting in a remarkable expansion of dendritic cells in the treated mice. The study demonstrated the usefulness of this systemic gene delivery approach for *in vivo* production of large amounts of gene product. Here, we extended the use of this simple gene delivery method for tumor treatment as a model to demonstrate its powerful potential for *in vivo* assessment of the therapeutic effect of a candidate gene. Moreover, we have demonstrated that this systemic gene transfer method could be useful for evaluating the potential toxicity of a candidate therapeutic gene/protein *in vivo*, which is usually difficult to achieve as large amounts of target protein or very high gene expression is needed. Using the IL-12 gene as an example, we showed that this gene transfer method resulted in a toxicity profile (including liver enzyme profile, serological profile, and immunological profile) similar to that of rmIL-12 (Lui *et al.*, 2001). With its advantages of being simple, minimally toxic, highly efficient, and economical, the hydrodynamics-based gene delivery method could become a powerful tool for the study of unknown gene functions or toxicity *in vivo*. Using this gene delivery method to perform a comprehensive *in vivo* study of potential therapeutic genes before launching a full-scale preclinical or clinical study would be very helpful.

Many recombinant proteins are being evaluated for their therapeutic effect in animal models, at great expense. Although the gene delivery method used in our study cannot be translated to clinical use at the present time (because of its relative invasiveness), the utility of the method for assessing the potential therapeutic effect of a candidate recombinant protein has nevertheless been demonstrated. This is because the gene product had *in vivo* activity comparable to that of the recombinant protein. Moreover, this efficient gene transfer method avoids the purification, reconstitution, stability, and contamination prob-

lems associated with the use of recombinant proteins. Information obtained from this type of gene transfer study could be beneficial for clinical trials of recombinant proteins. Besides, a therapeutic recombinant protein could be modified and improved via its gene construct and *in vivo* screening for the therapeutic effects of the modified recombinant protein could be easily assessed by simple delivery of the modified gene, using this method.

ACKNOWLEDGMENTS

We thank Genetics Institute (Cambridge, MA) for the generous gift of rmIL-12, and Dr. Stanley F. Wolf for invaluable advice. We also appreciated helpful discussions with Drs. Song Li, Feng Liu, and Mark Whitmore. This work was supported by the American Cancer Society, and by NIH grant 1R1AI43196-01 to Louis Falo and NIH grant CA 74918 to Leaf Huang.

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Received for publication September 15, 2000; accepted after revision November 27, 2001.

Published online: December 20, 2001.